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DEVELOPMENT AND VALIDATION OF REVERSE-PHASE HPLC METHOD FOR ESTIMATION OF KETOCONAZOLE IN BULK DRUG

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ABSTRACT

A simple, accurate rapid and precise RP-HPLC method has been developed and validated for determination of ketoconazole in bulk drug. The RP-HPLC separation was achieved on Promosil C-18, (250 mm, 4.6 mm, 5 μ m) using mobile phase water : acetonitrile : buffer ph 6.8 (51:45:4 v/v) at flow rate of 1.0 ml/min at ambient temperature. The retention times were 2.713 min. for ketoconazole. Calibration plots were linear over the concentration range 1-50 μ g/ml. Quantification was achieved with photodiode array detection at 238 nm over the concentration range of 1-50 μ g/ml. The method was validated statistically and applied successfully for the determination of ketoconazole. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for the routine determination of ketoconazole in bulk drug.

Keywords: Ketoconazole, Acetonitrile, Water, Buffer, HPLC, Method development, Method validation, Revers-phase HPLC method.

INTRODUCTION

Ketoconazole is Chemically Cis-1-acetyl-4-[4-[2-(2,4-dichlorophenyl)-2H-imidazolyl methyl]-1,3-dioxolan-4-yl] methoxy] phenyl]- piperazine is a topical as well as systemic antifungal agent. Ketoconazole is practically insoluble in water; sparingly soluble in strong acid, soluble in strong bases. It is an imidazole derivative with molecular weight 531.44.^{1,2} It inhibits cytochrome P450 dependent lanosterol C14 demethylase, which is responsible for production of ergosterol, a necessary component in fungal cell wall synthesis.^{3, 4} KT is a weak base with pKa values of 2.94 and 6.51.⁵ Ketoconazole is an

antifungal drug approved by the US FDA in 1981. Only a few analytical methods for the determination of the drug in biological samples and in the presence of other drugs have been reported.⁶⁻¹⁰

MATERIALS AND METHODS

All the reagents used were of HPLC grade and analytical grade and were purchased from Merck Chemicals, India. Reference standard of Ketoconazole was supplied as gift sample from Sun Pharmaceutical Laboratories Limited, Mumbai with purity of 99.987%.

- Preparation of buffer solution

Mix 7.601 gm of KH_2PO_4 in 1000mL of water. To 1000mL of 1.452 gm K_2HPO_4 solution, add 4.8 gm of sodium chloride anhydrous. Sonicate to dissolve.

- **Preparation of Mobile phase**

The mobile phase is prepared by mixing Water: Acetonitrile: Buffer pH 6.8 (51:45:4). Filtered and degas it

- **Chromatographic Run**

Load the standard solution of Ketoconazole in the injector, enter the HPLC parameters as per (Table1), save the method, inject and run for 10min.

- **Standard preparation of Ketoconazole**

Accurately weigh and transfer about 20mg of Ketoconazole working standard into 100mL volumetric flask and about 70 mL of diluents, sonicate to dissolve, dilute to volume with diluents and mix. Filter the solution through 0.45 μm .

- **Preparation of system suitability solution**

Accurately weigh and transfer about 10mg of working standard into 100ml volumetric flask. Add 25mL of 0.1N HCl and 25mL of Diluent. Sonicate to dissolve. Keep the sample at about 80 °C. For 4 hours. Use this solution as system suitability solution.

- **Preparation of placebo solution**

Accurately weigh and transfer powdered content of placebo equivalent to 100mg of Drug X into 100 mL volumetric flask. Add about 70 mL diluent and sonicate for about 15 min. dilute to the volume and mix. Filter the solution through 0.45 μm filters.

- **Preparation of diluent**

Use mobile phase as diluent.

As shown in Figure-3 Mobile phase = Water: Acetonitrile: Buffer pH 6.8 (51:45:4).

Method validation

The developed method was validated according to ICH guidelines. Standard calibration curve were prepared in the mobile phase with 5 concentration ranging from 1-50 $\mu\text{g}/\text{ml}$ for

ketoconazole is injected in to HPLC system keeping the injection volume constant. The peak area was plotted against the corresponding concentration to obtain the calibration graphs. To study the reliability and suitability of developed method, recovery experiments were carried out at three levels 80, 100 and 120%. Known concentration of Commercial tablet is spiked with known amounts of ketoconazole. At each level of the amount six determinations were performed and the results obtained were compared with expected results. Recovery for pharmaceutical formulations should be within the range 100 \pm 5%. The percent R.S.D of individual measurements was also determined. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentration of ketoconazole were analysed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision). The repeatability of sample application and measurement of peak area for active compounds were expressed in terms of percent RSD.

All chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional excipient peaks. Marketed formulation were analysed to determine the specificity of the optimized method in the presence of common tablet excipients. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from single to noise ratio. LOD and LOQ were calculated using $3.3\sigma/s$ and $10\sigma/s$ formulae, respectively. Where σ is the standard deviation of the peak areas and s is the slope of the corresponding calibration curve. To evaluate robustness of HPLC method a few parameters included variation of flow rate, percentage of buffer in the mobile phase, and pH of mobile phase.

RESULT AND DISCUSSION

The retention time of Ketoconazole was 2.713 min. The peaks are well separated with a resolution of 2.235 and Tailing 1.586. The

mobile phase comprises of The mobile phase is Buffer pH 6.8 (51:45:4) was selected as optimized mobile phase, because of the high purity, asymmetry, proper tailing, high area and low R_t value at same concentration as compared to other trail mobile phase. Furthermore, the stability of the drug in the mobile phase were also studied and result indicating that the drug Ketoconazole was found to be stable during the storage time of 48 hr (Table 2).

Linearity of the method was investigated by serially diluting the working standard to give a concentration range of 1-50 $\mu\text{g/ml}$ and 20 μl from this was injected. The flow rate was maintained at 1.0 ml/min. temperature of column was kept ambient and the effluent was monitored at 238 nm. Calibration curve was constructed by plotting concentration against peak area. The method was validated for linearity, precision, accuracy, specificity, limit of detection and limit of quantification as per ICH guidelines. All parameters are validated as per ICH guidelines.

Assay of tablets of Ketoconazole were performed. Twenty tablets of each company of strength 200 mg were weighed and ground to a fine powder. A quantity of tablet powder equivalent to 10 mg of Ketoconazole was transferred to 10 ml volumetric flask, dissolved and diluted with acetonitrile and water mixture to obtain 1 mg/ml. The solution was sonicated for 15 minute and filtered through 0.45 μm membrane filter. The solution was further diluted to obtain concentration 10 $\mu\text{g/ml}$. Peak area of the above prepared tablet solutions of Ketoconazole were measured by using above mentioned chromatographic conditions and the amount of Ketoconazole were found from regression equation. Optimum condition of mobile phases was investigated in the development of an HPLC method suitable for analysis in the bulk drug. These included Water: Acetonitrile: Buffer (50:20:30) (% v/v), Water: Acetonitrile: Buffer (60:20:20), Water: Acetonitrile: Buffer (60:25:15), Water: Acetonitrile: Buffer (65:25:10), Water: Acetonitrile: Buffer (55:45:10), and Water:

Acetonitrile: Buffer pH 6.8 (51:45:4). The same solvent mixture was used for extraction of the drug from the formulation containing excipients.

The linear regression data showed a good linear relationship over the concentration range of 1-50 $\mu\text{g/ml}$ as summarized in (Table 3). The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were found by scanning the solution of Ketoconazole having different lower concentrations and the LOD and LOQ were found to be 0.5 and 1 $\mu\text{g/ml}$ indicates that method is sensitive (Table 3). The intraday and interday precision were determined by analyzing standard solution of Ketoconazole at three different concentration levels (6, 8, 10 $\mu\text{g/ml}$). The % RSD for intraday and interday precision was found to be 0.257 – 0.712% and 0.480-1.080% respectively which indicate that method is precise (Table 3). Repeatability of the method was studied by injecting 10 $\mu\text{g/ml}$ solution of Ketoconazole for six times and peak area was measured and % RSD was calculated which was found to be 0.195 shows repeatability of the method (Table 3). Accuracy of the method was evaluated by standard addition method in which appropriate portion of stock solutions of Ketoconazole were spiked into blank placebo matrix to produce concentrations of 80 100 and 120% of theoretical concentration. The mean recovery of spiked samples obtained was in range of 99.06 to 101.03 reveals no interference of excipients and shows that method is accurate. The proposed validated method was successfully applied to determine Ketoconazole in tablet form. The results obtained for tablets of Ketoconazole were comparable with the corresponding labeled amounts (0.5 mg/tab) (table 4). Robustness of the method was estimated by changing the mobile phase composition (3 \pm 3), wavelength \pm 1 nm, injection volume (20 \pm 2 μl), column temperature (40 \pm 3 $^\circ$) and RSD values for all these changes calculated were less than 2 indicate that proposed method is robust. The proposed RP-HPLC method was accurate, precise, sensitive and rapid. The

method also can be extended for the routine analysis of Ketoconazole in tablet dosage form.

CONCLUSION

It is thus concluded that the proposed method is new, simple, cost effective, accurate, safe, free from pollution and precise and can be

successfully employed in the routine analysis of these drugs in pharmaceutical dosage forms. The proposed method shall prove equally effective to analyze Ketoconazole in the corresponding drug sample and may prove to be of great importance in pharmaceutical analysis.

Table1: HPLC instrumentation & chromatographic conditions for Ketoconazole

S. No.	Parameters	Description
1.	Instrument	A HPLC instrument (Younglin series) with Model Acme-9000
2.	Column	Promosil C-18, (250 mm, 4.6 mm, 5 μ m)
3.	Mobile Phase	Different mobile phase used for Trial 1 to 6
4.	Flow Rate	1.0 mL/minute
5.	Detection wavelength	238 nm
6.	Injection Volume	10 μ L
7.	Auto Sampler Temperature	5°C
8.	Run Time	10minutes

Table2: Stability of the Ketoconazole in the optimized mobile phase

S. No.	Storage conditions	Mean area \pm SD (At zero hrs)	SE	Mean area \pm SD (At 48 hrs)	SE
1.	Room Temperature (25 \pm 0.5 0C)	256754.3 \pm 2654.85	3143.7	250032.5 \pm 3033.44	2567.53
2.	Refrigerator (4 \pm 0.5 0C)	2576455.3 \pm 2787.76	3342.6	251254.1 \pm 3834.23	2654.37

*Concentration of drug 10 μ g/ml in mobile phase.

Table3: Stastiscal Data & Regression Equation for Ketoconazole

Sr. No.		Value
1.	λ_{max} (nm)	238
2.	Correlation coefficient (r ²)	0.999
3.	Regression equation	Y=0.67965X-3476
4.	Intercept (a)	03476
5.	Slope (b)	0.67965
6.	Limit of detection (LOD μ g/ml)	0.131
7.	Limit of quantification(LOQ μ g/ml)	0.463
8.	Linearity	1 – 50

9.	Accuracy%	99.06 to 101.03
10.	Repeatability (RSD, %, n=6)	0.195
11.	Precision (RSD, %), Interday (n=6)	0.257 – 0.712%
12.	Intraday (n=6)	0.480-1.080%

Table4: Results of analysis of commercial tablets of Ketoconazole

Tablet Formulation	Label claim (mg)	% Label claim estimated*(Mean ± S.D.)	% Coeff. of Variation	Standard error
I(KETOTAB)	200	100.435 ± 1.243	1.421	0.543
II(KETOZOL)	200	99.754 ± 1.509	1.543	0.703
III (KETONA)	200	99.246 ± 1.427	1.332	0.681

*Average of six determinations

Table5: System Suitability Test Parameters for Ketoconazole

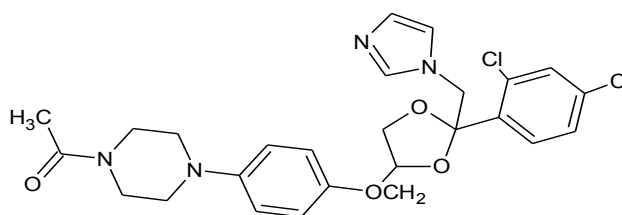
S. No.	Parameters	RP-HPLC method
1.	Retention time, min	2.713±0.356
2.	Tailing factor	1.586±0.274
3.	Asymmetry factor	1.123±0.315
4.	Theoretical plates	5987±0.9807
5.	Resolution	2.235±0.543

Result: The retention time of Ketoconazole was 2.713. The peaks are well separated with a resolution of 2.235 and Tailing 1.586.

Table6: Recovery studies of commercial tablets of Ketoconazole

Tablet Formulation	Label claim (mg)	Drug added (mg)	% Label claim estimated*(Mean ± S.D.)	% Coeff. of Variation	Standard error
I(KETOTAB)	200	100	99.816 ± 1.513	1.456	0.743
II(KETOZOL)	200	100	99.102 ± 1.397	1.437	0.574
III (KETONA)	200	100	100.288 ± 0.863	0.787	0.485

*Average of six determinations

**Figure1:** Chemical Structure of Ketoconazole

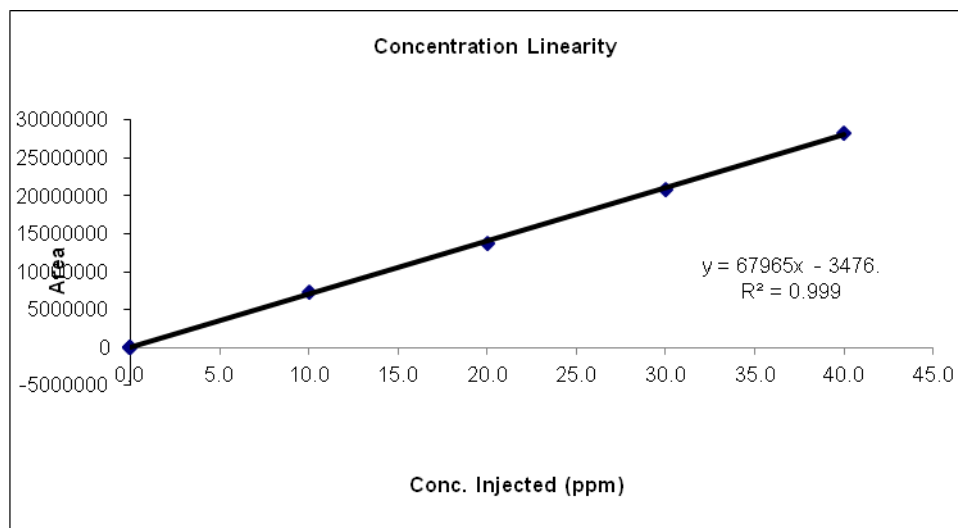


Figure2: Standard Curve of Ketoconazole

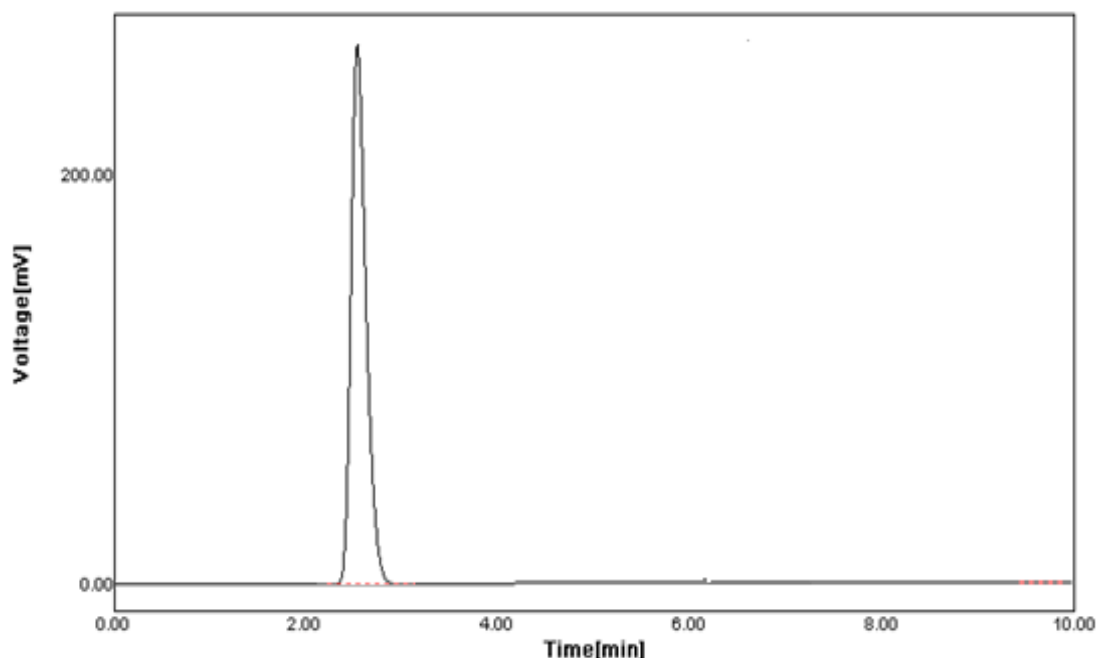


Figure3: Representative chromatograms of standard solution of Ketoconazole

Mobile Phase: Water: Acetonitrile: Buffer pH 6.8 (51:45:4)

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